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Stable gene amplification in the chromosome f *Bacillus subtilis*

(Recombinant DNA; antibiotic resistance; kanamycin, chloramphenicol; direct repeats)

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SUMMARY

We constructed five different structures, consisting of a genetic marker flanked by directly repeated sequences 2–4 kb long, in the *Bacillus subtilis* chromosome. When a selective pressure was applied amplification of the marker and one of the repeats was observed in all cases. Amplification was not detected with two markers which were not flanked by the repeated sequences. The maximum amplification level observed with the different structures varied between 5 and 50. The size of the most amplified structure corresponded to 7.5% of the chromosome. Amplification was stable upon growth of cells under non-selective conditions. Each copy of an amplified gene was expressed with equal efficiency. These results indicate that chromosomal gene amplification may be useful for constructing genetically engineered *B. subtilis* strains.

INTRODUCTION

Gene amplification has been observed in several prokaryotes such as Gram-negative *Proteus mirabilis* (Rownd and Michel, 1971; Rownd et al., 1973), *Escherichia coli* (Mottes et al., 1979; Normark et al., 1977; Chandler et al., 1979; Albertini et al., 1982), *Salmonella typhimurium* (Gutterson and Koshland, 1983) and Gram-positive *Streptococcus pneumoniae* (Clewell et al., 1975; Vasseghi and Claverys, 1983), *Streptomyces fradiae* (Fishman and Hershberger, 1983) and *Bacillus subtilis* (Young, 1984). It occurred most often when a selectable gene flanked by directly repeated DNA sequences was present on a plasmid (Peterson and Rownd, 1983) or on a chromosome (Gutterson and Koshland, 1983; Young, 1984). Amplifiable structures of this type may arise naturally, as when a gene happens to be flanked by two IS sequences (Hashimoto and Rownd, 1975; Clewell et al., 1975; Mattes et al., 1979) or be constructed artificially (Peterson and Rownd, 1983; Gutterson and Koshland, 1983; Young, 1984). In several reported instances the amplifiable structure was somewhat different, the selectable gene itself being a part of a directly repeated rather than of a unique sequence (Edlund and Normark, 1981; Albertini et al., 1982). This type of structure may arise by spontaneous duplication of bacterial genetic material (Anderson and Roth, 1977). Amplified structures

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Abbreviations: Ak, amikacin; 4'-ANT, 4'-adenosyl-nucleotidyl transferase; Ap, ampicillin; AU, amplification unit; Cm, chloramphenicol; EtdBr, ethidium bromide; kb, kilobases or kilobase pairs; Km, kanamycin; ^{R}, resistance; To, tetracycline.

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were never found to be stable in a recombination-proficient organism, i.e., the level of amplification decreases in the absence of selective pressure (Hashimoto and Rownd, 1975; Anderson and Roth, 1977; Normark et al., 1977; Yagi and Clewell, 1980; Vasseghi and Claverys, 1983; Spies and Laufs, 1983).

In the present work we describe gene amplification in the *B. subtilis* chromosome. We constructed five different amplifiable structures, consisting of two different selectable genetic markers and three different repeated sequences. Amplification was observed in all situations. This observation confirms and extends previous reports, where a single marker and a single repeat were used (Young, 1984; Sargent and Bennett, 1985) and indicates that amplification can occur in the *B. subtilis* chromosome whenever an amplifiable structure arises. We furthermore examined the stability of amplified structures and found no decrease of the level of amplification during prolonged growth in the absence of selective pressure, although the host bacterium was proficient in recombination. This unusual feature makes chromosomal gene amplification a potentially useful process for genetic engineering of *B. subtilis*.

MATERIALS AND METHODS

(a) Bacterial strains and plasmids

These are listed in Table I. The relevant constructions are described below.

(b) Enzymes

Restriction enzymes, DNA ligase and *E. coli* DNA polymerase were commercial preparations obtained from Boehringer (Mannheim, F.R.G.), Amersham (U.K.) or Genofit (Geneva, Switzerland) and were used according to suppliers' instructions. 4'-ANT activity was determined as described by Sadaie et al. (1980).

(c) DNA

Chromosomal DNA was prepared by CsCl centrifugation of lysozyme–SDS treated bacteria (Harris-Warwick et al., 1975). Plasmid DNA was prepared by CsCl–EtdBr centrifugation (Clewell and Helinski, 1969) or hydroxylapatite column chromatography (Colman et al., 1978) of appropriate lysates.

(d) Induction of competence and transformation

Competent *E. coli* and *B. subtilis* cells were prepared as described by Dagert and Ehrlich (1979) and Niaudet and Ehrlich (1979). 10–100 μg Ap/ml, 5–10 μg Tc/ml, 15–20 μg Km/ml and 25 μg Cm/ml were used to select *E. coli* transformants and 3 μg Cm/ml to select *B. subtilis* transformants. Km^R *B. subtilis* transformants could not be selected directly but could easily be identified in congression experiments (Niaudet et al., 1984).

(e) Hybridization

[^{32}P]DNA probes were prepared by nick translation as described by Rigby et al. (1977). Southern (1975) analysis and dot hybridization (Thomas, 1980) were performed as described.

(f) Strain constructions

Plasmids used for strain constructions are shown in Fig. 1. Strain construction scheme is shown in Fig. 2, the structures constructed in Fig. 3. Phenotype and Southern analysis of the strains gave the expected results for all strains (data not presented).

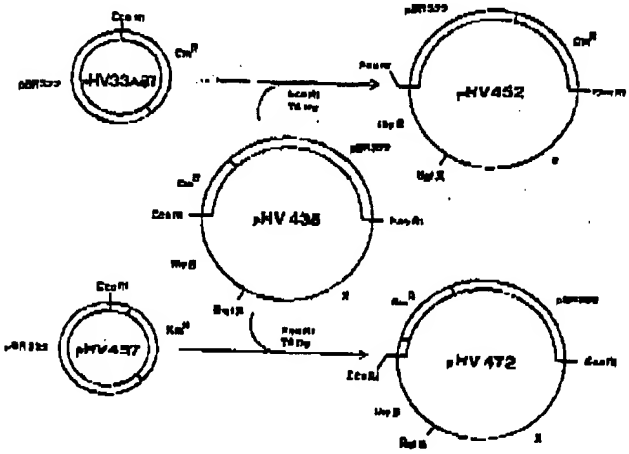

Fig. 1. Construction of plasmids carrying segments *thyB* and *X* of the *B. subtilis* chromosome.

TABLE I

List of strains and plasmids

Bacterial strains	Genetic markers[a]	Origin or reference
E. coli		
HVC45	*thrA1 leu-6 thi-1 lacY1 sm21 supE44 hsdR rpsL*	R. Davis
B. subtilis		
SB202	*trpC2 tyrA1 aroB2 hisH2*	P. Schaeffer
HVS246	SPβc2	S. Zahler
α	*tyrA1 aroB2 hisH2 trpC2 ins*(pHV452) *del*(*ilvA*)	Niaudet et al. (1984)
δ	*tyrA1 aroB2 hisH2 ins*(pHV472) *del*(*ilvA*)	this work
A	*tyrA1 aroB2 hisH2 ins*(pHV452) *del*(*ilvA*) *ins*(pHV457) *dup*(pBR322Δ81)	this work
B_T	*tyrA1 aroB2 hisH2 ins*(pHV472, *thyB*) *dup*(*thyB*) *dup*(*X*)	this work
B_X	*tyrA1 aroB2 hisH2 ins*(pHV472, *X*) *dup*(*X*) *dup*(*thyB*)	this work
D	*tyrA1 aroB2 hisH2 ins*(pHV472) *del*(*ilvA*) *ins*(pHV33Δ81) *dup*(pBR322Δ81)	this work
E	*tyrA1 aroB2 hisH2 ins*(pHV472, *X*) *dup*(*X*) *dup*(*thyB*) *ins*(pHV33Δ81) *dup*(pBR322Δ81)	this work

Plasmids	Nature or construction	Reference
pBR322	Cloning vector	Bolivar et al. (1977)
pC194	Natural isolate	Iordanescu (1975)
pUB110	Natural isolate	Gryczan et al. (1978)
pHV32	Tc^R revertant obtained in vivo from pHV14	Primrose and Ehrlich (1981)
pHV33	Hybrid between pC194 and pBR322	Primrose and Ehrlich (1981)
pHV33Δ1	*Bam*HI-cleaved pHV33 eroded by BAL 31	Niaudet et al. (1984)
pHV33Δ81	*Bam*HI-cleaved pHV33 eroded by BAL 31	Dagert et al. (1984)
pHV438	Hybrid between pHV32 and segments *thyB* and *X* of the *B. subtilis* chromosome	Niaudet et al. (1982)
pHV452	Hybrid between pHV33Δ81 and segments *thyB* and *X* of the *B. subtilis* chromosome	Niaudet et al. (1984)
pHV457	*Sau*3A segments I and IV of pUB110 inserted in the *Bam*HI site of pBR322	this work
pHV472	Hybrid between pHV457 and segments *thyB* and *X* of the *B. subtilis* chromosome	this work

[a] Genetic modifications due to insertion of plasmids into the chromosomes of *Bacillus subtilis* are described as follows (Niaudet et al. 1984): (1) *ins* followed by bracketed plasmid name denotes the inserted plasmid. When the insertion of the plasmid may occur in different chromosomal regions, the site of integration is indicated after the name of the plasmid. (2) *del* or *dup* followed by bracketed gene or sequence name and number denote deletions or duplications, respectively, resulting from plasmid insertion. (3) pBR322Δ81 represents the pBR322 sequences present in pHV33Δ81.

RESULTS

(a) Amplification of the Km^R gene

(1) Duplicated pBR322 sequences

The amplification unit present in the strain A consists of a Km^R gene and the sequences originating from pBR322 (Fig. 3). Amplification should give rise to bacteria carrying more than one copy of the Km^R gene, and therefore more resistant to Km than the parental strain. The proportion of bacteria resistant to different Km concentrations was determined by spreading a culture of strain A on the appropriate media (Table II). A unique copy of the gene conferred resistance to 1 μg Km/ml. A small fraction of th bacterial population was resistant to higher antibiotic concentration.

Bacteria resistant to different Km concentrations were isolated by cultivating strain A cells in appropriately supplemented liquid media. In a typical experiment 10^7 bacteria/ml were inoculated in L broth containing 10 μg Km/ml. The number of bacteria resistant to that antibiotic concentration, doubled each 20 min, as determined by plating aliquots of the culture on media supplemented with 10 μg Km/ml. This growth rate was the same as that of parental

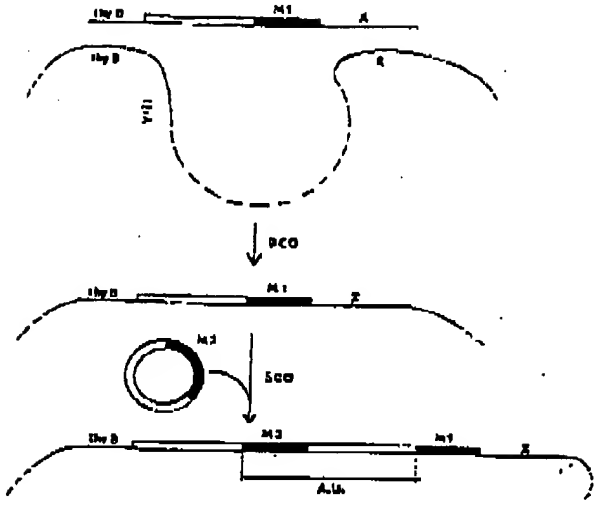

Fig. 2. Schematic representation of the construction of strains harboring duplications in their chromosome. Double and thin lines represent characterized plasmid or chromosomal DNA; respectively a dashed line represents uncharacterized chromosomal DNA; black boxes: genetic markers, which are indicated by M1 and M2. DCO, double crossing-over; SCO, single crossing-over.

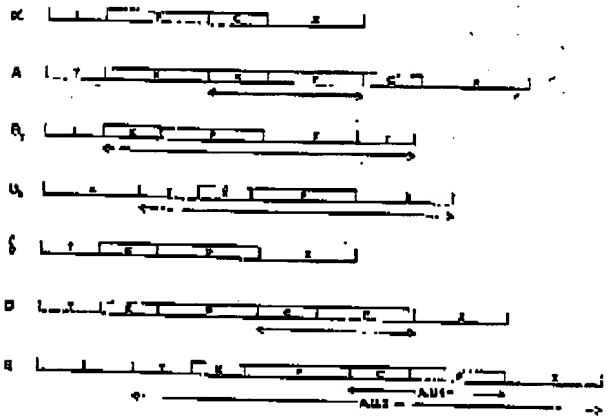

Fig. 3. Schematic representation of the *thyB* region of *B. subtilis* strains containing plasmids in their chromosome. Double and thin lines represent sequences of plasmid and chromosomal origin, respectively. C refers to Cm^R gene, K to Km^R gene, P to pBR322 sequences, T to *thyB* gene, *X* to a segment of unknown genetic content, A.U., amplification unit. Double-headed arrows delimit amplification units. Strains α and δ were constructed by a DCO integration of pHV452 and pHV472, respectively, into the chromosome of SB202, strains A and D by a SCO integration of pHV457 and pHV33Δ81 into the chromosome of α and δ, respectively, strains B_T and B_X by a SCO integration of pHV452 into the *thyB* or *X* region of the SB202 chromosome, respectively; strain E by a SCO integration of pHV33Δ81 into the B_X chromosome. SCO and DCO modes of integration are depicted in Fig. 2.

Fig. 4. Gene amplification in type A bacteria. 2 μg of chromosomal DNA extracted from bacteria grown at different Km concentrations (these are indicated above the lanes) were digested with *Cla*I and run on a 0.5% agarose gel in Tris·EDTA–borate buffer supplemented with 0.5 μg EtdBr/ml. *Hin*dIII-cleaved DNA was used as a size marker (not shown).

TABLE II

Proportion of bacteria resistant to increasing Km concentrations in cultures of strains A, B_T and B_X

Km concentration (μg/ml)	Plating efficiency[a]		
	A	B_T	B_X
0	1	1	1
1	0.99	1	1
2	1.0×10^{-4}	0.5	3.6×10^{-4}
4	5.6×10^{-6}	1×10^{-2}	2×10^{-5}
8	1.9×10^{-6}	2×10^{-6}	1×10^{-6}

[a] Compared to colony count in the absence of Km (= 1).

bacteria in an antibiotic-free medium, as determined in a control experiment. This indicates that exposure to Km did not induce the resistance, but that the more resistant bacteria were being enriched by growth in the selective medium.

We isolated bacteria resistant to 2, 2.5, 3, 4, 5, 8 and 10 μg Km/ml, and analysed their chromosomal DNA by electrophoresis after cleavage with *Cla*I. A strong fluorescent band was visible above the background of weak fluorescent bands in each DNA except that of the parental strain (Fig. 4). This shows that amplification took place, a segment of the same size being released by *Cla*I from each amplification unit.

The level of amplification was determined by densitometry (see footnote b in Table III). It increased from one copy in the parental strain to a plateau of about 30 in the strain resistant to 5 μg Km/ml (Fig. 5). These results show that the initial increase of the resistance to Km (until 5 μg Km/ml) is due to gene amplification, whereas the further increase may be due to other factors. A similar conclusion was reached by analysing DNA extracted from strain A bacteria resistant to much higher concentrations of Km (up to 320 μg/ml, Table III). The level of amplification determined by densitometry and dot hybridization did not exceed 30. High resistance was therefore not due to gene amplification, and can possibly be attributed to accumulation of mutations which renders cells impermeable to Km, such as those described previously for *E. coli* resistant to another aminoglycoside, streptomycin (Bryan and Van den Elzen, 1976).

TABLE III

Level of amplification in bacteria resistant to kanamycin

Km concentration (μg/ml)	Amplification level	
	Hybridization[a]	Densitometry[b]
1	1	ND
10	24	20
20	35	35
40	23	23
80	34	30
160	ND[c]	26
320	22	26

[a] Dot hybridization was used.

[b] Densitometry was performed on a negative of a photograph such as that shown in Fig. 4. The level of amplification was determined by comparing the area under the peak corresponding to amplified DNA segment with areas measured for control samples which contained the same amount of chromosomal DNA devoid of the amplified segment and a known proportion of a reference plasmid. The duplicate measurements differed by less than 10%. This method was used throughout this work to determine the level of amplification, unless stated otherwise.

[c] not determined.

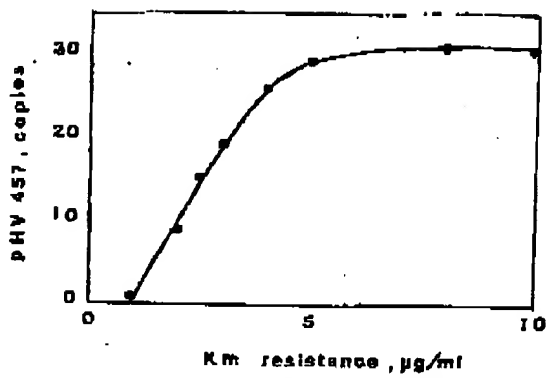

Fig. 5. Relationship between the copy number of pHV457 and the level of Km resistance of the strain A.

The resistance of bacteria harboring 1 or 30 copies of the amplification unit to different aminoglycosides was compared by the disc inhibition method. It increased in most cases with the increased gene number in a manner analogous to that observed for Km. Ak, however, was an exception, since only a low resistance was displayed by both strains, which suggests that the enzyme has a low affinity for that antibiotic. Starting with bacteria containing 30 copies of the amplification unit we isolated bacteria resistant to 8, 16 and 32 μg Ak/ml, and found that they contain 53, 46 and 51 copies of the amplification unit, respectively. This result illustrates that (i) amplification above the level of 30 may be achieved by using an adequate selection; (ii) a plateau of amplification is again encountered. It is interesting to note that bacteria resistant to Ak could be isolated from the cells containing a single amplification unit, but that they did not carry amplified structure. They were presumably permeation mutants of the type discussed above.

(2) Duplicated B. subtilis *sequences*

The amplification unit in strains B consisted of the Km^R gene, pBR322 and two sequences deriving from the *B. subtilis* chromosome, *thyB* and *X* (Fig. 3). In strains B_T and B_X duplication of *thyB* and *X*, respectively, should allow amplification. The pro-

portion of B_T and B_X bacteria resistant to different Km concentrations is shown in Table II. The former bacteria were more resistant than the latter to doses up to 4 μg Km/ml, a very small fraction of both being resistant to 8 μg Km/ml.

Bacteria resistant to 10 μg Km/ml were isolated by growth in liquid medium and the level of gene amplification was determined. B_T bacteria carried 5 copies of the amplification unit, while B_X bacteria carried 20 copies. These values were not higher in bacteria resistant to increased Km concentration (up to 20 mg Km/ml in the case of B_X). These results are analogous to those obtained with the strain A and show that amplification can occur in strains B_T and B_X. An inverse correlation between the level of amplification and the resistance conferred by a unique gene copy (judged by the fraction of bacteria resistant to a given Km concentration, Table II) is observed for the two B strains. The reasons for the variation of expression of the Km^R gene are not clear at present.

(b) Amplification of the Cm^R gene

The amplification unit present in the strain D consists of a gene encoding resistance to Cm and sequences originating from pBR322 and is thus very similar to that present in the strain A (Fig. 3). The strain carrying a single copy of the amplification unit was resistant to 5 μg Cm/ml. We have isolated bacteria resistant to 10, 25 and 50 μg Cm/ml by growth in liquid medium and repeatedly failed to isolate bacteria resistant to 100 μg Cm/ml or more.

The level of amplification in the different strains was determined after cleaving the extracted DNA with *Cla*I. This enzyme releases a 1.5-kb segment from the Cm^R gene and no sequences of that size from the *B. subtilis* chromosome, which allows measuring a low amplification level. No amplification was detected in bacteria resistant to 5 and 10 μg Cm/ml. Bacteria resistant to 25 and 50 μg Cm/ml carried 2.5 and 7 copies of the amplification unit. This shows that gene amplification occurs in the strain D.

(c) Amplification of Km^R and Cm^R genes

Two amplification units were present in the strain E. The first (AU1) is composed of the Cm^R gene and pBR322, the second (AU2) contains the entire AU1 Km^R gene and *B. subtilis* sequences *thyB* and ... (Fig. 3). In this situation the cells selected for an increased number of Km^R genes are expected to contain an increased number of the Cm^R genes while the cells selected for an increased number of the Cm^R genes need not contain an increased number of the Km^R genes.

Cells resistant to 25 μg Cm/ml and 10 μg Km/ml appeared with the frequency of 3.3×10^{-6} and 2.5×10^{-5}, respectively, in a culture of strain E grown in antibiotic-free medium. Cells grown in the medium supplemented with 10 μg Km/ml were resistant to both 10 μg Km/ml and 25 μg Cm/ml. This indicates that all sequences present within an amplification unit amplify at the same time. Surprisingly, cells grown in the medium containing 25 μg Cm/ml also became resistant to both antibiotics. This result suggests that the AU2 is amplified preferentially to AU1 in the strain E. The reasons for that preference are not known.

(d) Amplification requires duplicated sequences

Km^R and Cm^R genes could be amplified when flanked by repeated DNA sequences. To determine whether duplication is essential for amplification we made use of strains α and δ in which the two genes are not flanked by duplications (Fig. 3) and which are resistant to 1 μg Km/ml and 5 μg Cm/ml, respectively. We isolated bacteria resistant to 10 μg Km/ml from the strain α and found no amplification in their chromosome by either densitometry or Southern hybridization. We failed to isolate bacteria resistant to 10 μg Cm/ml from the strain α. Bacteria resistant to 5 μg Cm/ml contained no amplified structure. These results indicate that gene amplification occurs in the *B. subtilis* chromosome only when duplicated sequences are present.

(e) Stability of amplified sequences

To examine the stability of amplified sequences we cultured Km-resistant cells of the type A (Fig. 3) which carried 30 copies of the amplification unit for 150 generations in a Km-free liquid medium. Plating efficiency of the culture on a medium containing 7.5 μg Km/ml remained 100% throughout that period. In a control experiment we determined that

cells containing 15 copies of the amplification unit plate on that medium with an efficiency of 0.1%. If in our plating assay 100% efficiency could not be distinguished from that of 90%, the frequency of loss of 15 copies of the amplification unit (giving cells containing only 15 copies) was less than 10^{-3} per generation (the fraction F of cells maintaining the original number of copies is given by $F = 1 - (1 - p)^{n+1}$ where p and n stand for frequency of loss per generation and number of generations). This observation indicates that the amplified structures are maintained stably in cells of the type A. It was confirmed by DNA analysis of cells cultured for 150 generations in the absence of Km, which contained 28 copies of the amplification unit, as determined by densitometry. This value is not significantly different from 30, determined for cells at the outset of growth in the Km-free medium. A similar result was obtained with cells of the type B (Fig. 3) which carried 20 copies of the amplification unit. No loss of resistance was observed by the plating assay after 20 generations of growth in a Km-free medium.

(f) Expression of the amplified Km^R gene

Km^R gene used in this work derives from plasmid pUB110 and encodes 4'-ANT. We determined the enzyme activity in crude extracts of type A bacteria containing the amplified gene and found that it was linearly proportional to the gene number (not shown). It is interesting to note that strains, resistant to very high levels of Km (20 mg/ml) and containing 30 copies of the gene, did not produce more enzyme than the strains resistant to 5–10 μg Km/ml, which also contained 30 copies of the gene. This shows that the high resistance was not due to the enhanced expression of the Km^R gene, and supports the conclusion drawn from DNA analysis that it may be due to the presence of unrelated mutations.

DISCUSSION

We observed amplification of five structures consisting of an antibiotic resistance gene flanked by duplicated DNA sequences in the *B. subtilis* chromosome. No amplification was observed if the resistance gene was not flanked by duplications. This result, together with those of other authors (Young, 1983; 1984; A. Albertini and S. Galizzi, 7th European Meeting on Transformation, Paris, 1984; Sargent and Bennett, 1985) indicates that amplification may occur in *B. subtilis* whenever an adequate structure arises in its chromosome.

Different structures could be amplified to different levels. Maximal amplification was observed with a gene specifying 4'-ANT flanked by repeated pBR322 sequences (strain A). Cells carrying up to 50 copies of the gene were isolated by a two-step enrichment procedure, selecting first for growth in Km, then in Ak. The amplified structure represented 7.5% of the chromosome (the unit of amplification and the chromosome being 6 kb and 4000 kb, respectively). 20 and five copies of the same gene were found when it was present within a different amplification unit (strains B_X and B_T), the size of amplified structures being 7.5% and 2.5% of the chromosome, respectively. The level of resistance was nevertheless identical in the three situations. This suggests that the Km^R gene may be expressed with a different efficiency within different amplified structures. The reasons for this are not clear at present. Within the same amplified structure each gene copy appeared to function with the same efficiency, as judged by the fact that in cells of the type A the amount of 4'-ANT synthesized was proportional to the number of genes present.

The level of Cm resistance (50 μg/ml) and the amplification of the Cm acetylase gene (7 copies) observed here were somewhat lower than those previously reported (50–100 μg Cm/ml; 7–20 copies; Young, 1984; A. Albertini and S. Galizzi, 7th European Meeting on Transformation, 1984; Sargent and Bennett, 1985). This may be due to differences between strains and/or amplifiable structures used in different experiments. Further experiments may allow differentiation between these alternatives. The lower level of amplification of the Cm^R than that of the Km^R gene may be due to the fact that the acetyl-coenzyme A, used for modifying the former antibiotic, becomes limiting in the cell and, therefore, renders selection of cells carrying a higher number of genes impossible (Foster, 1983).

Although amplified structures are generally not maintained stably in recombination-proficient bacteria, we observed high stability of the amplified structures studied here. This feature, which remains

to be explained, indicates that chromosomal gene amplification may become useful for constructing genetically engineered *B. subtilis* strains.

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